Circular Epstein-Barr Virus Genomes of Reduced Size in a Human Lymphoid Cell Line of Infectious Mononucleosis Origin

ALICE ADAMS, GUNNAR BJURSELL, CHRISTINE KASCHKA-DIERICH, AND TOMAS LINDAHL

Departments of Tumor Biology, Biochemistry, and Chemistry, Karolinska Institute, 104 01 Stockholm, Sweden

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Circular Epstein-Barr virus (EBV) DNA molecules have been purified and characterized from a human lymphoid cell line derived from a case of heterophile antibody-positive, blood transfusion-induced infectious mononucleosis, 883L. The circular EBV DNA in three cell lines obtained by transformation of human umbilical cord blood leukocytes with a strain of EBV originally derived from 883L was also studied. As estimated from sedimentation velocity data and electron microscopy, the circular EBV DNA molecules are 10 to 15% smaller than either the circular EBV DNA previously found intracellularly in several other types of EBV-transformed cells or the linear EBV DNA present extracellularly in virus particles. In addition, the EBV-transformed cord blood cell lines studied here differed from other EBV-transformed cells in that integrated virus DNA sequences could not be detected.

Human lymphoid cell lines regularly contain several copies of the Epstein-Barr virus (EBV) genome (26, 39), and both integrated virus DNA sequences and circular, nonintegrated virus DNA molecules have been found in such EBV-transformed cells (2-4, 15, 20). A similar situation with respect to the physical state of the intracellular virus DNA has subsequently been observed in a number of other systems, e.g., in polyoma virus-transformed rat cells (30), in quail tumor cells persistently infected with avian sarcoma virus (11), and in Herpesvirus saimiri-transformed marmoset cells (F. J. Werner, G. W. Bornkamm, and B. Fleckenstein, Abstr. Xth Meet. Eur. Tumour Virus Group, 1976, p. 173). It would appear that EBV is no longer a unique example of a tumor virus that can be carried as an episome, although most mammalian and avian cells transformed by tumor viruses only contain integrated virus DNA.

The circular EBV DNA molecules present in EBV-transformed cells can be purified free from cellular DNA and used to characterize the latent virus genomes (20). In the present work, we have studied the intracellular EBV DNA present in a human lymphoid cell line derived from a heterophile antibody-positive case of post-transfusion mononucleosis, 883L (6, 25). The EBV DNA in cell lines established by transformation of human umbilical cord blood leukocytes with the B95-8 variant of EBV (23), which originates from 883L, was also investigated. EBV particles from the 883L line have malignant potential in animals, since they have been shown to induce lymphomas after injection into cottontop marmosets (35).

There are a number of interesting differences between the intracellular EBV DNA in the lines studied here and the EBV DNA in previously investigated cells. First, the circular EBV DNA molecules found here are smaller than those present in several other types of EBV-transformed cells, and they are also smaller than the linear EBV DNA molecules present in virus particles. Second, the human umbilical cord blood leukocytes recently transformed with EBV studied here contain circular virus DNA molecules, but in contrast to other EBV-carrying cell lines investigated they do not contain readily detectable amounts of integrated virus DNA sequences.

MATERIALS AND METHODS

Cells and virus. All cell lines were propagated as stationary suspension cultures at 37°C in RPMI-1640 medium (Grand Island Biological Co.) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. The 883L, B95-8, P3HR-1, and Raji cell lines were obtained from G. Klein, Department of Tumor Biology, Karolinska Institute. The cell lines cb35B1, cb35B3, and cb43B11 were established by immortali-
zation of human umbilical cord blood leukocytes with EBV preparations derived from B95-8 cells. These three lines were established by M. Dalens, Department of Tumor Biology, Karolinska Institute, following the procedures of Miller and Lipman (23). The lines were at passage 30 to 50 when the present work was performed. Cells were screened for "early-antigen" production according to Klein et al. (17).

EBV particles were isolated from the spent media of P3HR-1 cultures (12) or [3H]thymidine-labeled B95-8 cultures (23) and used for the preparation of virus DNA as described (1).

**DNA fractionation.** The general experimental techniques used in this laboratory for the fractionation of cellular DNA of high molecular weight from human lymphoid cells by gradient centrifugation, and the subsequent identification of EBV DNA sequences by nucleic acid hybridization, have recently been described in detail (20). Briefly, cells from actively growing cultures were washed with phosphate-buffered saline and lysed at pH 9 in a Sarkosyl- and EDTA-containing buffer. After treatment with Pronase, the lysate was adjusted to a DNA concentration of 10 µg/ml, supplemented with CsCl and a trace amount of Klebsiella pneumoniae [3H]DNA as an internal density marker, and centrifuged in 19-ml aliquots to apparent equilibrium. Fractions (0.4 ml) were collected through a large hole in the bottom of a tube with a closed-system collection device in order to avoid shearing forces. After localization of (i) the density marker by radioactivity determinations of an aliquot of each fraction and (ii) the cellular DNA by measurements of absorbancy at 260 nm, the fractions from one gradient were usually used for nucleic acid hybridization tests, and the material from the other gradients was used for further fractionation.

For glycerol gradient centrifugation, fractions from CsCl gradients of density 1.712 to 1.724 g/cm³ were pooled, supplemented with a trace quantity of phage T4 [32P]DNA as a size marker, dialyzed free from CsCl, and concentrated by packing the dialysis bags in dry polyethylene glycol 6000. The DNA was then dialyzed again, and 1-ml aliquots were applied to 36-ml neutral glycerol gradients. Fractions (1 ml) were again collected through a large hole in the bottom of the centrifuge tube. The size marker was localized by determination of the 32P radioactivity of aliquots of the fractions. EBV DNA sequences in gradient fractions were then localized by DNA-complementary RNA (cRNA) hybridization. The EBV cRNA was obtained by transcription of EBV DNA from P3HR-1-derived virus particles with Escherichia coli RNA polymerase in the presence of [α-32P]CTP. After alkali denaturation of the DNA, individual fractions were applied to 13-mm membrane filters and incubated with 1 ng of EBV [32P]cRNA (1.6 × 10⁶ cpm) in 0.3 ml of 0.9 M NaCl-0.09 M trisodium citrate-50% formamide for 4 days at 45°C. After washing and RNase treatment, the amount of filter-bound 32P was determined.

**Electron microscopy.** The DNA sedimenting at 90 to 110S in glycerol gradients was dialyzed free from glycerol and X-irradiated as described (20). A dose of 1,000 rad was used, with the DNA in a Tris- and histidine-containing buffer, in order to introduce an average of two single-strand interruptions per covalently closed circular EBV DNA molecule. Samples were then mounted for length measurements, together with the open circular form of phage PM2 DNA as a reference, by microdiffusion onto parlodion-coated grids from a cytochrome c-containing buffer (19, 20). After staining with uranyl acetate and shadowing with platinum-palladium, micrographs were taken in a Philips electron microscope 301. Contour lengths were measured by optically projecting micrographs onto a Scriptographic digitizer coupled to a Compucorp 445 statistician calculator programmed with a fully smoothed-length calculation program.

**RESULTS**

**Cell lines.** The 883L cell line is an EBV producer at a low level (25). Thus, the 883L cells investigated here contained 0.5% cells that were positive for early antigen, indicating that they had entered an abortive lytic-virus cycle. Miller and Lipman have used EBV particles released by the 883L line to immortalize murine cells, and an excellent EBV producer line, B95-8, has been obtained in this fashion (23). Virus released from the latter line has been used to transform human umbilical cord blood leukocytes, and resulting EBV-carrying lines are usually virus nonproducers that contain multiple copies of EBV DNA per cell (26). The three separate umbilical cord blood cell lines of this type studied here all had 10 to 15 EBV genome equivalents per cell (5) and did not produce EBV (<0.01% early-antigen-positive cells). The physical state of the EBV DNA in these three lines and in the parent line 883L was investigated. The B95-8 line was not studied, since the techniques used are not well suited for the characterization of latent EBV DNA in cell lines containing a large proportion of actively virus-producing cells.

**CsCl gradient centrifugation.** EBV DNA has a higher density (57 to 58% guanine-cytosine base pairs; 33) than most of human DNA (~42% guanine-cytosine base pairs), so intracellular nonintegrated EBV DNA can be partly purified from host DNA by CsCl density gradient centrifugation. When high-molecular-weight DNA preparations (molecular weight \([M_w] \approx 150 \times 10^6\)) from the cb35B1, cb35B3, and cb43B11 lines were fractionated by this technique, centrifugation profiles similar to that in Fig. 1 were obtained. The peak of EBV DNA, as localized by nucleic acid hybridization experiments with the individual gradient fractions, was found at the density characteristic of EBV DNA from B95-8-derived virus particles. The present results differ slightly from those
previously obtained with the extensively studied Raji cell line, where the peak of intrinsic EBV DNA of Raji cells was found at a 0.002-g/cm³-lighter density than EBV DNA from virus particles (4).

The small amount of intracellular EBV DNA found at a density of less than 1.710 g/cm³ (Fig. 1) at least partly consisted of material tailing from the main peak of virus DNA, but this material could also have contained virus DNA sequences covalently bound to cellular DNA. The apparent presence or absence of integrated virus DNA sequences can be further studied by rebanding the DNA in CsCl, and such experiments were performed with the DNA in the density region of 1.700 to 1.710 g/cm³ (Fig. 2). The 883L line clearly contained EBV DNA sequences that remained at a low density also in rebanding experiments (Fig. 2). Similar data have previously been obtained for the EBV DNA present in Raji cells and in Burkitt lymphoma tumor biopsies, and the occurrence of such viral DNA sequences has been shown to indicate integration (2, 4, 15). However, the EBV-transformed cord blood lines differ from previously investigated cells in that no integrated EBV DNA sequences could be detected (Fig. 2).

Glycerol gradient centrifugation. The fractions from CsCl gradients enriched in EBV DNA (e.g., fractions 20 through 24 in Fig. 1) were further characterized by neutral glycerol gradient centrifugation. The EBV DNA in all cases sedimented as two distinct components (Fig. 3 and Table 1), indicating the presence of covalently closed circular DNA and open circular DNA. In comparison with our previous results on the circular EBV DNA in lymphoma-derived material (15) or in cell lines established from normal individuals without lymphoproliferative disease (C. Kaschka-Dierich, L. Falk, G. Bjursell, A. Adams, and T. Lindahl, manuscript in preparation), the sedimentation coefficients observed here were 4 to 5% lower (Table 1). Using a relation of $s_1/s_2 = (M_1/M_2)^{0.38}$ (9), this would correspond to a molecular-weight difference of 10 to 15% between the infectious mononucleosis-derived EBV DNA studied here.

Fig. 1. Neutral CsCl density gradient centrifugation of high-molecular-weight DNA from cb35B3 cells (density = 1.700 g/cm³). K. pneumoniae ($^{3}H$)DNA (density = 1.717 g/cm³) was added as a reference, and the DNA was centrifuged in a Spinco 60 Ti rotor at 33,000 rpm and 21°C for 65 h. The gradients were linear over the entire region of interest (density = 1.68 to 1.75 g/cm³) as determined by refractive index measurements. Symbols: (●) Cellular DNA absorbancy at 260 nm; (○) K. pneumoniae ($^{3}H$)DNA; (▲) EBV DNA sequences (hybridization with EBV [32P]cRNA).

Fig. 2. Rebanding experiments in neutral CsCl gradients. Cellular DNA of density 1.700 to 1.710 g/cm³ (e.g., fractions 26 through 29 in Fig. 1) were pooled and recentrifuged under the same conditions: (Top) DNA from cb35B3 cells; (bottom) DNA from 883L cells. Symbols as in Fig. 1.
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and intracellular EBV DNA from other sources.

Kolodner et al. (18) have derived an empirical relationship between the \( s_{20, w, Na^+} \) and the molecular weight of large, open circular DNA molecules from measurements on chloroplast DNAs isolated from several different plants. This relation, \( s = 0.05587 \times M_w^{0.38} \), yields values of approximately \( M_w = 117 \times 10^6 \) for the open circular EBV DNA from Raji cells and \( M_w = 105 \times 10^6 \) for the open circular EBV DNA from the cord blood lines studied here. The molecular-weight value for the open circular EBV DNA from Raji cells is 10% higher than previous estimates (20). This may mean either that the equation of Kolodner et al. (18) yields molecular-weight values that are slight overestimates or that the value used for the sedimentation coefficient of our reference, T4 DNA, is too high. With regard to the latter point, all our sedimentation data have been determined relative to T4 DNA in co-sedimentation experiments. The \( s_{20, w, N_a^+} \) for T4 DNA used here, 61.8S, is an average value of several determinations performed in different laboratories (9), but there is presently no general agreement on the exact sedimentation coefficient of T4 DNA.

In any case, the difference in size between intracellular EBV DNA circles from different sources found here should be relatively little affected by such calibration errors in the absolute data.

Molecular weights may also be estimated from the sedimentation coefficients of the covalently closed circular DNA molecules, using the equation \( s = 0.0312M_w^{0.44} \) of Hobom and Hogeness (13). This yields \( M_w = 100 \times 10^6 \) for the circular EBV DNA from Raji cells and \( M_w = 89 \times 10^6 \) for the circular EBV DNA from 883L cells.

The sedimentation coefficient of the linear EBV DNA present in virus particles derived from the B95-8 cell line was also measured by co-sedimentation of the EBV (HJ)DNA and T4 (14C)DNA. The EBV DNA sedimented as a symmetrical peak at 0.95 times the rate of T4 DNA, corresponding to a sedimentation coefficient for the EBV DNA of 58 to 58S. This is the same value as that previously found (9) for EBV DNA from P3HR-1-derived virus particles. Taken together, the data confirm an earlier study by Pritchett et al. (31) on the identical size of the EBV DNAs in virus particles derived from the B95-8 and P3HR-1 cell lines. If the EBV DNA from B95-8-derived virus particles had been 10 to 15% smaller than the virus DNA from P3HR-1-derived particles, it would have been expected to have a sedimentation coefficient of about 58S, i.e., 0.90 times the value for T4 DNA (9). This was clearly not the case.

**Electron microscopy.** The high-density DNA sedimenting at 90 to 110S in glycerol gradients (fractions 10 through 13 in Fig. 3) was recovered, dialyzed free from glycerol, exposed to a low dose of X-irradiation in order to convert covalently closed circles to an open circular form, and investigated by electron microscopy. The open circular form of phage PM2 DNA was used as an internal reference in all experiments. Large circular DNA molecules of uniform size were found (Fig. 4), accounting for...
approximately 2% of the total DNA in the fraction. It has previously been shown that such DNA circles are absent in human lymphoid cell lines that do not contain EBV DNA (20). The contour lengths (in micrometers) of 28 EBV DNA molecules were: 44.96, 45.15, 45.68, 45.72, 45.75, 45.95, 45.96, 46.14, 46.15, 46.33, 46.49, 46.75, 46.83, 46.84, 46.86, 46.87, 46.94, 47.07, 47.09, 47.11, 47.12, 47.13, 47.23, 47.58, 47.59, 48.02, and 48.47. The average contour length was 46.7 μm, with a standard deviation of 1.8%. The PM2 DNA had a contour length of 3.18 μm (standard deviation, 1.5%), so the circular EBV DNA in the cb35B3 line was 14.7 times longer than PM2 DNA. If the molecular weight of PM2 DNA is taken as 6.4 × 10^6 (29) and the number of base pairs per unit length of DNA is assumed to be independent of base composition, the molecular weight of the EBV DNA circles from cb35B3 cells is 94 × 10^6.

The intracellular circular EBV DNA from Raji cells, as well as the linear EBV DNA from virus particles derived from the B95-8 cell line, have previously been shown to have a contour length 16.6 times that of PM2 DNA, corresponding to an approximate molecular weight of 106 × 10^6 (20, 31). There was not a single circular EBV DNA molecule of that large size in the material from the cb35B3 line (Table 2). Moreover, out of 35 circular EBV DNA molecules from Raji cells, not a single DNA molecule as short as the circles studied here was detected (20). It seems clear that the cb35B3 line contains EBV DNA circles that are about 12% shorter than the EBV DNA present intracellularly in the Raji cell line or extracellularly in EBV particles. These contour length data are in excellent agreement with the sedimentation velocity data, which indicated a size difference of 10 to 15% between the EBV DNA circles studied here and EBV DNA from other sources.

The circular EBV DNA present in the 883L cell line was also investigated by electron microscopy. Due to low DNA concentration, only eight molecules were measured, but they all had contour lengths 14.5 ± 0.3 times that of PM2 DNA. No size differences were observed between the EBV DNA circles in the 883L cell line and those in the umbilical cord blood cells transformed with the B95-8 variant of EBV, which originates from 883L cells. Thus, there is no indication here that the 883L-derived virus has been altered by its passage through B95-8 marmoset cells. It is noted that New World nonhuman primates such as marmosets do not carry simian EBV-like viruses (8).

**DISCUSSION**

Several different strains of herpes simplex type 1 or human cytomegalovirus have been isolated and shown to have closely related but nonidentical DNA sequences (16, 36). Moreover, it has been reported that different strains of herpes simplex type 1 contain DNA molecules of slightly different sizes in the virus particles (38). It has been more difficult to study the question of strain differences between EBV isolates by biochemical techniques, since there is no known lytic system for this virus. However, two good virus-producer cell lines have been available, P3HR-1 and B95-8, and they clearly release different types of EBV. Besides the biological differences between these two strains of EBV (22, 24), their DNA sequences are only 85 to 95% homologous, as judged from DNA reassociation kinetics studies and from analysis of DNA fragments obtained by restriction enzyme cleavage (31, 37). Partial denatur-
atation mapping experiments have revealed that the B95-8-derived EBV DNA studied here has an unique sequence that is not circularly permuted (G. W. Bornkamm and H. Delius, personal communication). In a different approach, Pagano and co-workers (28) have determined the final extent of DNA reassociation achieved in hybridization experiments with EBV DNA from virus particles and cellular DNA from various types of EBV-transformed cells. It was found that not all the virus DNA sequences present in the probe, radioactively labeled virus DNA from the Burkitt lymphoma-derived P3HR-1 lines, were present in tumor biopsies from nasopharyngeal carcinoma patients. By the same technique, it has recently been shown that the EBV DNA present in several lymphoid cell lines derived from infectious mononucleosis patients is not completely homologous to the EBV DNA in P3HR-1-derived virus particles. After prolonged incubation at 66°C of P3HR-1-derived EBV (3H)DNA with cellular DNA from the lines, only 60 to 75% reassociation was observed, indicating that "an extensively deleted, defective EBV genome is retained in lymphocyte lines established from infectious mononucleosis or that the cell lines harbour different strains of EBV" (27). However, a higher degree of homology between P3HR-1-derived EBV DNA and the intracellular EBV DNA present in infectious mononucleosis-derived cell lines has been found by Pritchett et al. (32) in similar experiments.

The present results, compared with our earlier data on the properties of the circular EBV DNA in Raji cells (20), are most easily interpreted as reflecting differences between different strains of EBV. The smaller size of the intracellular, circular EBV DNA than the linear DNA from corresponding EBV particles observed here suggests that the linear virus DNA has a large terminal redundancy of about 12% of the total length of the virus genome, which is lost on circularization and regenerated on linearization. This situation is by no means unique, since there are several bacteriophages which contain linear DNA that is converted to a smaller, circular DNA form intracellularly (14, 21). Although it has been technically difficult to study terminal redundancy in EBV DNA directly, other herpesviruses have been shown to have terminally redundant DNA (10, 34), and channel catfish herpesvirus DNA has recently been shown to have a large terminal sequence redundancy of about 10^6 daltons (P. Sheldrick, N. Berthelot, and S. Chousterman, personal communication). Thus, it is not unreasonable to propose a similar large terminal redundancy in the DNA of the EBV strain studied here. The DNA reassociation kinetics data of Pritchett et al. (31) indicated that EBV DNA from B95-8-derived virus particles had a 12 to 15% lower sequence complexity than P3HR-1-derived EBV DNA, and instead contained a reduplicated DNA sequence to yield the same total genome length; such a situation would agree well with the data presented here. Another possibility consistent with the present results would be that the 883L-derived EBV genome is reversibly dissociated into two circular molecules of 94 x 10^6 and 12 x 10^6 daltons intracellularly, in the same fashion as found for certain bacterial plasmids (7), and that we have only recovered the larger DNA circle here. A third possibility is that in addition to the circular viral DNA molecules, 883L cells contain integrated EBV DNA molecules of 106 x 10^6 daltons, which serve as templates during the production of virus particles.

In contrast to previous work on latent EBV DNA in lymphoma-derived cells (2, 4, 20) or the situation observed in the 883L cell line (Fig. 2), we could not detect EBV DNA sequences with the properties of integrated EBV DNA in the EBV-transformed cord blood cell lines investigated (Fig. 2). These data certainly do not exclude the possibility that there were, nevertheless, some integrated virus DNA sequences present. For example, the EBV DNA might be integrated as fragments that were not detected in the hybridization tests because they were present only in small quantities or because the corresponding sequences were not represented in the EBV cRNA probe used, or the EBV DNA might be integrated into an unusually guanine-cytosine-rich region of the cellular DNA and consequently not registered as integrated DNA by the density analysis used. On the other hand, we feel that the present data show that there was less than one complete EBV genome integrated into cellular DNA of typical average density (1.700 g/cm^3). Most of the 10 to 15 EBV genome equivalents found in the cord blood cell lines were removed in the first CsCl gradient centrifugation step, and the presence of an integrated virus genome would have been expected to be reflected as a detectable amount of EBV DNA banding at a lower density than free EBV DNA in the rebanding experiments. This was not observed (Fig. 2). It is conceivable that upon immortalization of human lymphoid cells with EB virus, the EBV DNA is primarily converted to circular virus DNA molecules, whereas stable integration of virus DNA is a much later event. It may also be that EBV DNA is carried in different ways in different types of host cells. We plan to investigate the physical state of the EBV DNA in other cord
blood cell lines and to follow the lines studied here to see whether integrated virus DNA sequences will be detected after prolonged passage in culture.

EBV has been found to be consistently associated with several different human disorders, and it is obviously of considerable interest to determine whether different strains of EBV can be related to various disease patterns. The circular EBV DNA found intracellularly in Burkitt lymphoma-derived tumor biopsies and cell lines, as well as the EBV DNA circles found in nasopharyngeal carcinoma tumor cells, are larger than the EBV DNA studied in the present work (15, 20). However, large EBV DNA circles of the same size as in the African Burkitt lymphoma-derived cells have also been found in two American lymphoblastoid cell lines, F-265 and NC-37, derived from the blood of normal individuals not suffering from either malignant disease or infectious mononucleosis (Kaschka-Dierich et al., in preparation), so there is clearly no simple relationship between the size of the intracellular EBV DNA and its association with malignant disease. It will be necessary to study the size of the latent EBV DNA in many different human lymphoblastoid cell lines from different sources in order to determine whether the presence of intracellular circular EBV DNA molecules of small size can be related to a specific clinical syndrome, to a specific cell type, or to other factors.

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LITERATURE CITED


